REMARKS/ARGUMENTS

Claims 40 and 41 have been amended as suggested. No new matter has been added, and no new issues have been raised. Should the Examiner consider these amendments to raise new issues, the Examiner is authorized to cancel Claims 40 and 41.

The obviousness rejection of Claims 18-19, 22, 25-36, and 39-41 as being unpatentable in view of Brohuit and Ecomer is traversed.

Claim 18, the sole independent claim, is drawn to a method of reducing at least one skin damage in a subject in need thereof (emphasis added). The Office, at 5 of the Official Action, asserts, that "any person would be in need thereof reducing skin damage." The Office's assertion is wrong; the "in need thereof" feature of Claim 18 is not described or suggested by the cited references, and the cited references cannot as a matter of law render obvious the present claims.

The "in need thereof" language used here has been approved by the Federal Circuit for inventions like present Claim 18 where the method requires the specific intent to effect the specific results stated in the preamble of the claim.

The case of Jansen v. Rexall Sundown Inc., 68 U.S.P.Q.2d 1154 (Fed. Cir. 2003) requires that the currently pending rejection be withdrawn. In *Jansen*, the claims were directed to methods of treating or preventing macrocytic-megaloblastic anemia comprising administering effective amounts of folic acid and vitamin B_{12} to humans in need thereof. *Jansen* at 1157. In interpreting these claims, the Federal Circuit ruled that the claims require the specific intent to achieve the claimed objective (treatment or prevention of macrocytic-megaloblastic anemia). Specifically, the Federal Circuit stated that:

> . . . the claim preamble sets forth the objective of the method, and the body of the claim directs that the method be performed on someone 'in need.' In both cases, the claims' recitation of a patient or a human 'in need' gives life and

> meaning to preambles. [Citation omitted]. The preamble is therefore not merely a statement of effect that may or may not be desired or appreciated. Rather, **it is a statement of the intentional purpose for which the method must be performed**. We need not decide whether we would reach the same conclusion if either of the 'treating or preventing' phrase or the 'to a human in need thereof' phrase was not a part of the claim; **together, however, they compel the claim construction arrived at by both the district court and this court**.

Jansen at 1158 (emphasis added).

Thus, according to the Federal Circuit, claims directed to methods of treatment to be performed on those in need of such treatment require the specific intent to effect such treatment.

Present Claim 18 is analogous to the claims in Jansen and is directed to a method of reducing at least one skin damage in a subject in need thereof ... (see Claim 18, *supra*). The cited references neither teaches nor suggests such specific intent: that is, the cited references do not describe or suggest administering the composition in Claim 18 to a subject in need thereof in need thereof in an amount sufficient to reduce the at least one skin damage in the subject in need thereof.... Nothing in the cited references would lead one skilled in the art to believe that the composition in Claim 18 could be administered for this express purpose. Indeed, the Office appears to concede this point by asserting, contrary to the holding of *Jansen*, that "any person would be in need thereof reducing skin damage." Thus, the cited references cannot render obvious present Claim 18 and the claims depending therefrom because they do not describe or suggest all of the claimed features. Withdrawal of the obviousness rejection is requested on this basis alone.

Additionally, Applicants traverse the obviousness rejection on the grounds that the Office has not made a proper obviousness rejection. The at least one skin damage of Claim 18 is selected from the following conditions induced by UV light: skin cancer, formation of pigmented spots, formation of freckles, formation of wrinkles, formation of verrucae, and formation of erythema. The cited references do not describe or suggest these species, as acknowledged at page 3 of the Official Action. Nevertheless, the Office at page 5 of the Official Action, asserts that "since the prior art discloses the composition can be used to treat UV induced skin damage, it would have been obvious to...attempt treatment utilizing the composition for the instantly claimed disorders since they all arise from the same origin." The Office's reasoning is flawed.

First of all, the Office has not specifically pointed out where Brouhit describes the genus skin damage caused by UV radiation. This is because Brouhit does not use the term skin damage caused by UV radiation. The Office assumes a genus not present in Brouhit. Further, the Office has provided no motivation for selecting the specifically claimed skin damage species from a (non-present) broad genus of UV induced skin damage and has shown no reasonable expectation of success in making the selection. For example, the Office has not shown the cited references are enabled for every species in the broad genus of UV induced skin damage, and this weighs against any reasonable expectation of success. Further, Brohuit provides no experimental support for any disease other than psoriasis, and refers to no additional references. Psoriasis is a suspected autoimmune disease,[1] and factors "that may aggravate psoriasis include stress, withdrawal of systemic corticosteroid, excessive alcohol consumption, and smoking."[2] "It has long been recognized that daily, short, non-burning

[1] See http://en.wikipedia.org/wiki/Psoriasis.
[2] Id.

exposure to sunlight helped to clear or improve psoriasis in some patients"[3] (emphasis added). Indeed, Wikipedia describes that: "It was during the early part of the 20th century that it was recognised that for psoriasis the therapeutic property of sunlight was due to the wavelengths classified as ultraviolet (UV) light" (emphasis added). Thus, far from causing psoriasis, UV light exposure to the skin can be therapeutic for treating psoriasis (emphasis added). Thus, treating psoriasis, a disease that can be treated by exposure to UV light waves, and the only disease state Brohuit may be enabled for, cannot possibly suggest, motivate or enable orally administering glycerol ethers to reduce the claimed conditions induced by UV light.

Further, the Office asserts, regarding the specific conditions of Claims 32-36, that "it would have been obvious...to have used the composition of Brohuit for the treatment of UV light induced skin damage since it is disclosed that ethers beneficially influence diseases depending upon epidermal growth" (see page 4 of the Official Action). The Office's reasoning is flawed. Brohuit is not enabled for reducing the Claim 18 UV light induced skin conditions. In direct contradiction to the Office's assertion that "ethers beneficially influence diseases depending upon epidermal growth," Applicants note a large number of studies have been carried out in the field of diseases depending upon epidermal growth, and to Applicants' knowledge, none of these studies suggests that reduction in the skin damages of Claim 18 depend on regulating epidermal growth. An exemplary article from these studies is enclosed with this paper (P.I. Chen et al, Journal of Biological Chemistry, 2009 – the Chen article). The Chen article does not describe or suggest reduction in skin damage as claimed in present Claim 18, even though the article is directed to epidermal growth factors.

Moreover, in making the obviousness rejection, the Office notes that Brouhit describes that "burns caused by intensive sunrays can be partly prevented by administration

[3] Id.

[of ethers]" (see Office Action page 3). Reduction of the Claim 19 skin conditions is not suggested by Brouhit's burns statement because the Office has filed to establish a linkage between burns caused by intensive sunrays and the Claim 18 skin conditions. Additionally, as part of not being enabled for partly preventing burns caused by intensive sunrays, Brouhit's burns statement does not specify how these burns can be partly prevented. The phrase "oral administration" at line 56, column 1 of Brouhit refers only to treatment of severe haemmorhoids, and severe haemmorhoids do not typically arise because of intense sunray exposure. Accordingly, Brohuit is not enabled for and does not describe or suggest, oral administration of glycerol ethers to prevent or reduce sunburn.

The Office has not made a proper *prima facie* case of obviousness. Withdrawal of the obviousness rejection is requested on this basis alone.

Finally, concerning the Office's assertion that Claims 40-41 are product by process claims, the amendments to Claims 40-41 moot this assertion.

Applicants submit the present application is now in condition for allowance. Early notification to this effect is earnestly solicited.

Respectfully submitted,

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RAB5 ISOFORMS DIFFERENTIALLY REGULATE THE TRAFFICKING AND DEGRADATION OF EPIDERMAL GROWTH FACTOR RECEPTORS

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Running title: Rab5A specifically regulates EGFR degradation pathway

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Ligand-mediated endocytosis is an intricate regulatory mechanism for epidermal growth factor receptor (EGFR) signal transduction. Coordinated trafficking of EGFR ensures its temporal and spatial communication with downstream signaling effectors. We focused our work on Rab5, a monomeric GTPase shown to participate in early stages of the endocytic pathway. Rab5 has three isoforms (A, B and C) sharing more than 90 % of sequence identity. We individually ablated endogenous isoforms in HeLa cells with siRNAs and examined the loss-of-function phenotypes. We found that suppression of Rab5A or 5B hampered the degradation of EGFR, whereas Rab5C depletion had very little effect. The differential delay of EGFR degradation also corresponds with retarded progression of EGFR from early to late endosomes. We investigated the activators/effectors of Rab5A that can potentially separate its potency in EGFR degradation from other isoforms and found that Rin1, a Rab5 exchange factor, preferably associated with Rab5A. Moreover, Rab5A activation is sensitive to EGF stimulation, and suppression of Rin1 diminished this sensitivity. Together with previous work showing that Rin1 interacts with ESCRT-0 complex (Hrs/STAM) to facilitate the degradation of EGFR (1), we hypothesize that the selective association of Rab5A and Rin1 contributes to the dominance of Rab5A in EGFR trafficking, whereas the other isoforms may have major functions unrelated to the EGFR degradation pathway.

Rabs are small-molecular-weight guanine nucleotide binding proteins (G proteins) specialized in regulating different stages of the intracellular membrane trafficking based on their subcellular localization and interacting protein scaffolds (2). The increasing number of Rab family members during evolution reflects ongoing specialization of membrane trafficking pathways. At least 63 Rabs have been identified in humans. Among these, Rab5 serves as the master regulator of the endocytic trafficking (3). It functions through recruitment of specific effector proteins involved in membrane tethering and docking (4-8). The demonstration that at least 20 cytosolic proteins specifically interact with active Rab5 highlights the complexity of the downstream regulation by this GTPase and raises the possibility that Rab5 might also manage other aspects of the endosome function (4,9). A common feature of the Rab family small GTPases is the existence of subgroups of structurally related isoforms sharing a high sequence identity (10). Rab5 subgroup has three isoforms (A, B, and C) (3). A large-scale mRNA expression profiling study from 79 human and 61 mouse non-redundant tissues shows distinct tissue distributions of the Rab5 isoforms, suggesting that the trafficking properties of the early endosomal network in developmentally distinct cell and tissue types is likely fine-tuned to fulfill sub-specializations of a given pathway (10). In fact, several lines of evidence indicate that Rab5 isoforms can be functionally different. First, Rab5A is found to be the only isoform transcriptionally up-regulated in response to treatment with cytokines like IL-4 or interferon (IFN-γ) in macrophages (11,12). Moreover, Rab5A but not Rab5C was found to be involved in the maturation of phagosomes containing *Listeria monocytogenes* (13). In primary hippocampal

cultures, treatment with DHPG, a group I metabotropic glutamate receptor agonist, leads to up-regulation of Rab5B but not Rab5A, thereby reducing N-methyl-d-aspartate (NMDA)-type glutamate receptor-mediated membrane current and cell death (14,15). Also, Rab5 isoforms can be differentially phosphorylated at a consensus motif in position 123 by distinct proline-directed Ser/Thr kinases *in vitro* (16).

Epidermal growth factor receptor (EGFR) is the prototype of Class I transmembrane receptor tyrosine kinase operating through activation of its intrinsic tyrosine kinase upon ligand binding. Activated EGFR stimulates numerous signal transduction pathways that mediate a wide spectrum of cell responses, including cell proliferation, differentiation and apoptosis (17,18). In order to regulate the strength and duration of the signaling, activated EGFR also initiates a negative-feedback mechanism that eventually leads to the removal of EGF-EGFR complexes from the plasma membrane by endocytosis (19). Previous studies have shown that over-expression of Rab5A enhances EGF-stimulated fluid-phase endocytosis and EGF-EGFR internalization; whereas dominant negative Rab5 represses these processes (20,21). In addition, dominant negative Rab5 substantially inhibits the degradation of EGFR (21). On the other hand, continual expression of constitutively active Rab5 causes a ligand-independent redistribution of EGFR into the intracellular vesicles (22). Interestingly, although expression of a constitutively active Rab5 shows no significant effect on EGFR levels (22), it enhances cell growth and receptor signaling (23).

In this study, we took advantage of the RNAi-silencing technique to individually knock down endogenous Rab5 isoforms in HeLa cells, so that we could examine the loss-of-function phenotypes more likely to capture their physiological activities. Our results indicate that suppression of Rab5A significantly impairs the degradation of EGFR; whereas Rab5C suppression has little effect. The delay of EGFR degradation elicited by the absence of Rab5A occurs after EGFR is internalized, since the rate of EGFR internalization is unaffected by suppression of a single isoform. In addition, we identified Rin1, a Vps9 domain-containing Rab5 exchange factor, to preferentially associate Rab5A, and selectively potentiate its activity in response to EGF stimulation.

EXPERIMENTAL PROCEDURE

Antibodies and Reagents- A mouse monoclonal anti-Rab5A antibody was received as a gift from Dr. A. Wandinger-Ness (University of New Mexico. Albuquerque, NM). Anti-Rab5B antibody was kindly provided by Dr. David B. Wilson (Washington University, St. Louis, MO). Anti-Rab5C antibody is obtained from Sigma Aldrich (Prestige Antibodies®, HPA003426). Human epidermal growth factor and monoclonal anti-EGFR (Ab5) antibody were obtained from CALBIOCHEM. Rabbit polyclonal anti-EGFR (1005) and monoclonal GFP antibody (sc-9996) was from Santa Cruz Biotechnology, Inc. Polyclonal GFP antibody was kindly provided by Dr. Phyllis Hanson (Washington University, St. Louis, MO). Monoclonal anti-V5 antibody was from Invitrogen. Rabbit polyclonal Hrs antibody was a gift of Dr. Tim McGraw (Cornell University). Monoclonal anti-EEA1 and anti-Rin1 antibodies were both from BD Transduction Laboratory. Anti-Cbl antibody (7G10) was from Upstate. Anti-Grb2 antibody was from Cell signaling. Anti-Ubiquitin antibody was obtained from Zymed Laboratories (Invitrogen).

Plasmid constructs- cDNA of Rab5A, B and C were subcloned into SalI/ BamHI sites of pEGFP-C1 (Clontech) and SacI/ BamHI sites of RFP-C3 expression vector (kindly provided by Arnold Hayer et. al., Swiss Federal Institute of Technology (ETH) Zürich, Switzerland). A Rin1 expression construct was prepared by subcloning full-length human Rin1 PCR product into the mammalian expression vector pcDNA3.1/V5-His TOPO TA (Invitrogen).

*siRNA construction and transfection-*The siRNAs against Rab5 isoforms were constructed and purified using the Silencer™ siRNA construction kit (Ambion, Austin, TX) as described. The sequences specific for human Rab5A are 5'-GAGTCCGCTGTTGGCAAATCA-3' (#84) and 5'-AACCAGGAATCAGTGTTGTAG-3' (#623), for human Rab5B are 5'-ACCCAGTCCGTTTGTCTAGAT-3'(#174) and 5'-AAGACAGCTATGAACGTGAAT-3' (#492),

for human Rab5C are 5'-ACCA ACACAGATACATTTGCA-3' (#309) and 5'-AATGAACGTGAACGAAATCTT-3' (#503), and for human Rin1 are 5'-AACAGTCTGAGACAACTGCTG-3' and 5'-AACATGTCCTGGAGAAGTCAT-3'. A scrambled siRNA (Ambion, Austin, TX) or siRNA designed against GFP was used as negative controls. The transfection of siRNA (20 nM final concentration) was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instruction.

Cell culture and transfection- HeLa, HEK293T, and DU145 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% bovine growth serum (Hyclone Laboratories) containing penicillin and streptomycin. The transfection was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instruction.

Immunoblotting and Immuno-precipitation- Immunoblotting analysis was conducted as described before (1). Briefly, cell lysates were prepared with the lysis buffer containing protease inhibitor cocktail (Sigma). The cell lysates were clarified by centrifugation prior to separation by SDS-PAGE. The resolved proteins were transferred to nitrocellulose membranes (Whatman Schleicher & Schuell, Florham Park, NJ) and then blocked in TBST containing 5% nonfat milk. The membranes were probed with primary antibodies and then HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and proteins were visualized by enhanced chemiluminescence detection reagents (Pierce). Immunoblot data were quantified by AlphaEaseFC 4.0 software (Alpha Innotech Corp. San Leandro, CA). For immuno-precipitation, the clarified cell lysates were incubated with primary antibodies and protein G-Sepharose (Sigma) overnight at 4 °C. The beads were washed extensively with lysis buffer and solubilized in SDS sample loading buffer.

EGFR Degradation assay- Control or experimental cells were serum starved and then treated with 100 ng/ml EGF and 25 μg/ml cyclohexamide for different times. At the end of each time point, cells were washed with PBS and then lysed in lysis buffer as indicated above. The lysates were subjected to SDS-PAGE and immunoblotting with appropriate antibodies.

*Biotinylation and Internalization of Cell Surface EGF Receptor-*Confluent HeLa cells pre-treated with siRNA were starved in serum-free medium. One set of experiment was kept un-stimulated, while the other two sets were stimulated with 100 ng/ ml EGF for 2 and 5 minutes. The internalization was stopped by washing with ice-cold PBS-Ca-Mg (PBS, pH 7.4, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$). Chilled cells were then incubated with biotin-labeling solution containing 0.5 mg/ml EZ-Link® Sulfo-NHS-LC-Biotin (Pierce) in PBS-Ca-Mg at 4 °C for 30 min. The reaction was quenched by washing the cells with ice-cold PBS-Ca-Mg containing 15 mM glycine. The cells were then washed again with PBS and lysed as described above. The biotinylated-EGFRs were purified using MagnaBind™ streptavidin beads (Pierce) according to the product instructions. Precipitated biotin-EGFR then were subjected to SDS-PAGE and immunoblot analysis. The rate of internalization is calculated by densitometry of EGFR bands.

Quantification of endogenous Rab5 isoforms- His-Rab5A, 5B and 5C recombinant proteins were purified from bacterial lysates. HeLa and DU145 cell lysates were prepared as described above. 5ug or 10 ug of total proteins from DU145 or HeLa cells respectively were subjected to SDS-PAGE alongside purified recombinant Rab5 isoform standards. Intensity of the protein standard bands was quantified with AlphaEaseFc 4.0 software and plotted against their concentration pre-determined with BCA assay in order to define the linear range of the signal. Band signals from cell lysates were then calculated to determine the ratio of different isoforms.

Immuno-fluorescent Microscopy- Cells grown on cover slips were fixed with 3% paraformaldhyde (Electron Microscope Sciences), permeabilized, and then blocked with goat serum. Following incubation with primary antibodies and then Alexa-Fluor594 or 488-conjugated secondary antibodies, the cover slips were mounted with Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA). Confocal microscopy was performed using a 63X objective and fluorescein

sets on a MRC1024 (Bio-Rad Laboratories). Uptake of Alexa-488-labeled EGF or Transferrin was performed by first starving cells in serum-free medium for 3 hours or 1 hour at 37°C. Cells were then incubated with cold serum-free medium containing 400 ng/ml fluorescein EGF or 40 μg/ml Transferrin for 1 hour at 4 °C. Endocytosis were initiated by replacing the lignad binding medium with pre-warmed medium. At the end of each time point, cells were rapidly chilled, washed, fixed with PFA and then subjected to immunostaining as described above.

Rab5 activation assay- cDNA of the Rab5-binding domain (R5BD, residues 739-862) of Rabaptin5-pGEX was a kind gift from Dr. Guangpu Li (University of Oklahoma Health Sciences Center). The resulting construct termed pGEX/Rabaptin-5(R5BD) was expressed in DH5α. GFP-Rab5 isoforms were expressed in HeLa cells for 24 hours. Cells were first starved and then treated with 100 ng/ml EGF for 5 minutes. Cells were subjected to lysis for 5 min in the lysis buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, and protease inhibitor). Aliquots of clarified lysates were incubated with GST-R5BD pre-bound to the glutathione-Sepharose-4B resin for 30 min at 4°C on a rotating mixer. The resin was subsequently rinsed with the lysis buffer, resuspended in SDS sample buffer and then subjected to SDS-PAGE and immunoblot analysis.

Statistical analysis- All experiments presented were repeated a minimum of three times. The data represents the mean ± SE. Student's t test was used to calculate statistical significance.

RESULTS

Depletion of Rab5A in HeLa and DU145 cells leads to delayed EGFR degradation

To study the functional differences of endogenous Rab5 isoforms, we designed siRNAs to specifically silence individual isoforms. At least two independent siRNAs were used to suppress each isoform. Immunoblotting with isoform-specific antibodies confirmed that the suppression was more than 90% from each siRNA, and the isoform-specific siRNAs did not cross-react with other isoforms, as the levels of the other two isoforms were unaffected when one isoform was silenced (Fig.1A). Following siRNA treatment, HeLa cells were assayed for EGFR levels at steady state. We found that cells depleted of Rab5A or 5B consistently exhibited increased level of total EGFR; whereas, loss of Rab5C showed very little effects (Fig.1A). The accumulation of EGFR in cells depleted of Rab5A or 5B suggests that the sorting of EGFR into degradation pathway is likely interrupted. Therefore, we examined the rate of EGFR degradation by stimulating HeLa cells with EGF for different times. The levels of EGFR at early time points (3 and 8 minutes) were not used for quantitative analysis, because we found that the immunoblot signals of EGFR were highly variable among experiments at these time points. The results (collected from 15, 30 and 60 minutes) showed that in HeLa cells, the initial rate of degradation was substantially reduced in Rab5A or 5B-depleted cells; however, between 45-60 minutes, the receptors eventually underwent degradation to an extent similar to the control cells (Fig.1B, C and S1). The degree of delay is more profound with Rab5A depletion than with Rab5B knockdowns. Of all three Rab5 isoforms, Rab5C suppression seems to have the least impact. This result suggests that the degradation of EGFR requires both Rab5A and 5B. It is possible that the two isoforms work in concert or sequence along the degradation pathway, so that only one of the two isoforms present is not sufficient to transport EGFR at normal rates. We also tested the rate of EGFR degradation in DU145 cells. DU145 is a prostate cancer cell line expressing EGFR at much higher level than HeLa cells. In agreement with what we found in HeLa cells, the depletion of Rab5A also leads to more delayed EGFR degradation in DU145 cells (Fig.1D). This finding not only verified that the function of endogenous Rab5 is critical for sorting EGFR into degradation pathway, but also provided new evidence indicating a preferential connection between endogenous Rab5A and the trafficking of EGFR.

Quantification of Rab5 isoforms in HeLa and DU 145 cells

Previously, Bucci *et al.* reported that HeLa cells express all three Rab5 isoforms with Rab5B to be more enriched than the other two (16). To test the possibility that the differential delay of EGFR trafficking caused by individual Rab5

isoform silencing corresponds to the relative isoform abundance, we examined the concentration of Rab5 isoforms in HeLa and DU145 cells using purified recombinant Rab5 isoform proteins as standards. Whole cell lysates from HeLa or DU145 cells were analyzed alongside Rab5 protein standards during SDS-PAGE. Following protein gel transfer, membranes were probed with isoform-specific antibodies and the intensity of the bands from protein standards was plotted against its concentration to demonstrate the linearity of the band signals (Fig.S2A and S2B) and the avidity of the isoform-specific antibodies. Using this method, we were able to estimate the ratio of endogenous Rab5A: 5B: 5C was approximately 1: 1: 2 in HeLa cells and 2: 3: 1 in DU145 cells (Fig.2 A and B). These data show that Rab5A is not the most abundant isoform in either cell line, yet it dominates the endocytic trafficking of EGFR.

Over-expression of Rab5A shows more potency in down-regulating EGFR

We next tested if over-expression of individual Rab5 isoforms has opposite effects on EGFR degradation. HeLa cells were transiently transfected with individual CFP-Rab5 isoforms overnight and EGFR degradation assays were carried out as described above. Our findings indicate that exogenously expressed Rab5A, but neither 5B nor 5C accelerated the degradation of EGFR in HeLa cells (Fig. 3A, B). In accordance with the biochemical analysis, immunostaining of total EGFR in cells transfected with RFP-Rab5 isoforms revealed that Rab5A-expressing cells have weaker EGFR signals, suggesting enhanced down-regulation (Fig. 3C, S3). We did, however, notice the degree of acceleration was relatively mild (Fig. 3A, B). Since the transfection efficiency of Rab5 isoform constructs generally does not exceed 80% in HeLa cells, it is possible that the acceleration of EGFR degradation is masked by untransfected cells. Alternatively, we reasoned that under our experimental conditions, endogenous Rab5 isoforms may have already maximized the rate of trafficking and sequestered the limiting factors participating in this process; therefore, excess Rab5 could not improve the transport much further. On the other hand, the finding with Rab5A expression did coincide with the outcome of its depletion, implicating Rab5A as the primary Rab5 isoform in the EGFR degradation pathway.

Depletion of individual Rab5 isoforms shows no significant effect on EGFR internalization

Next, to identify the sites where Rab5A and/or 5B are most active, we examined if loss of individual isoforms could interfere the internalization of EGFR. Control or Rab5 isoform siRNAs-treated HeLa cells were starved and then stimulated with EGF for the indicated times (Fig. 4A, B). EGFR remaining on the cell surface was biotin-labeled, precipitated by streptavidin beads and analyzed with immunoblotting. The data show that depletion of individual Rab5 isoforms did not inhibit the internalization of EGFR, whereas simultaneous depletion of all three isoforms delayed the internalization by ~50% (Fig. 4A, B). Similar results have been reported previously with ^{125}I-EGF based assay by Sorkin *et al.* (24). Similar to EGFR, the endocytosis of Transferrin (Tfn) receptors was not altered by silencing of individual Rab5 isoforms, while silencing all three isoforms together showed marked reduction of Tfn uptake (Fig. 4C, S4).These data suggest that Rab5 isoforms, though actively participating in the internalization step of receptor trafficking, are functional redundant at this stage.

Rab5A or 5B delayed the exit of EGFR from early endosomal compartments

After internalization, EGFR sorting into the late endosome and degradation in the lysosome are necessary to terminate receptor signaling. c-Cbl-mediated ubiquitylation has been shown to be essential for regulating these events and ensuring proper degradation of EGFR (25,26). Upon EGF stimulation, c-Cbl binds directly to the EGFR via Tyr-1045 and indirectly through the SH3 domain of Grb2, whose recruitment to the EGFR is mediated by phosphorylation at Tyr1068 and Tyr1086 of EGFR (27,28). We therefore asked whether the delayed degradaton of EGFR as a result of Rab5A or 5B depletion correlates with altered EGFR phosphorylation and ubiquitylation. By immunoprecipitating EGFR after stimulation for different periods of time and probing ubiquitylated EGFR with anti-ubiquitin antibody, we found that the relative levels of EGFR ubiquitylation over time is not altered by depletion of Rab5A or 5B (Fig. 5A). Consistently, depletion

of Rab5A or 5B did not suppress the phosphorylation of EGFR at Tyr-1045 or Tyr1068 (Fig. 5B, S5), nor did the silencing of Rab5 isoforms interfere the recruitment of c-Cbl or Grb2. However, it is unclear whether depletion of Rab5A or 5B alters the phosphoryaltion of other tyrosine or serine sites of the EGFR and/or obstructs its access to other key adaptors. Further studies will be needed to reveal a more detailed phosphorylation map of EGFR in response to Rab5 isoform silencing.

To further investigate how the loss of individual isoforms changes endosomal dynamics, we examined the subcellular distribution of EEA1, a FYVE-domain containing protein known to associate with the early endosomal compartment (4,6,29) and Hrs, that also localizes on early endosomal membranes and transfer cargos with ubiquitin moieties to downstream multivesicular body sorting machineries (30). In the mean time, the transport of EGFR was visualized by either indirect EGFR immunostaining or by using Alexa488-labeled EGF. Consistent with the immunoblotting data, we observed stronger EGF (or EGFR) signals in Rab5A or 5B-depleted cells (Fig.6). The association of EEA1 with membrane structures was not impaired by loss of individual Rab5 isoforms, nor was the distribution of Hrs and CD63, a late endosomal marker (Fig.6 and S6). Similar to control cells, the EGF-EGFR complex in isoform-silenced cells entered EEA1 positive compartments within 10 minutes (Fig.6A). At later time points, most EGFR containing vesicles was still EEA1 positive in Rab5A- or 5B- silenced cells, while a substantial subset of EGFR in control and Rab5C-depleted cells had exited from early endosomal compartments (Fig.6B and C). Similar results were obtained with Hrs co-immunostaining (Fig.S6A). 45 minutes after endocytosis, EGFR signals had almost completely disappeared from Hrs-positive compartments in control or Rab5C knock-down cells, but still partially co-localized with Hrs in Rab5A, or 5B knock-down cells (Fig.S6A). These results suggest that depletion of Rab5A or 5B slows down the progression of EGFR from early to late endosomal compartments, thereby causing the delay of EGFR degradation. Even so, Rab5A or 5B-silenced cells did eventually complete the degradation of EGFR, suggesting that they can still overcome this blockage presumably via residual activity from incompletely silenced Rab5 isoform, compensatory activities from Rab5 isoforms not targeted by siRNA or alternative mechanisms independent of Rab5.

Rin1 preferentially associates with Rab5A, which facilitates the activation of Rab5A in response to EGF stimulation

Previous studies have shown that Rab5 can be activated by HGF stimulation (31) and that active Ras, a consequential effector of EGFR signaling, potentiates the activity of Rab5A *in vitro* (32). Both studies suggest that the function of Rab5 can be regulated by receptor signaling. In an effort to better understand why Rab5A displays more potency in the EGFR degradation pathway, we tested if Rab5 isoforms are differentially activated in response to EGF stimulation. We took advantage of a Rabaptin-5 Rab5 binding domain (R5BD)-based GST pull down assay (33) to study the activation Rab5 isoforms *in vivo*. We found that after 5 minutes of EGF treatment, more Rab5A was bound to GST-R5BD, indicating more activation of Rab5A; whereas, Rab5B and Rab5C activation did not increase in response to EGF at this time point (Fig. 7). This result further supports the hypothesis that Rab5A is more potent in regulating the trafficking of EGFR as its activation is more responsive to the EGF stimulation. Our previous work shows that Rin1, a Vps9 domain-containing Rab5 exchange factor, relays EGFR signals to Rab5 activation (32). Also, Rin1 has an SH2 domain that couples Rab5 activation to the proximity of EGFR (34). Recently, we found that Rin1 works in concert with ESCRT-0 complex (STAM1/Hrs) in down-regulation of EGFR (1). These data make Rin1 a prime target in Rab5-mediated EGFR trafficking. Therefore, we tested if Rin1 shows any specificity towards Rab5 isoforms. Dominant negative (S34N), and GTP hydrolysis-deficient constitutively active (Q79L) forms of Rab5 isoforms were used to evaluate Rin1 specificity. We carried out a co-immunoprecipitation study with cells transiently co-expressing one of the Rab5 mutant isoforms along with Rin1. Characteristic of exchange factors, Rin1 binds preferentially to dominant negative Rab5, which is primarily GDP-bound. Interestingly, we found that Rin1 bound to Rab5A with higher affinity than to Rab5B or Rab5C (Fig. 8A). Another Rab5 exchange factor, GAPex-5

(human RME-6) (35), was also tested for Rab5 isoform specificities, and no apparent selectivity was found (data not shown). To determine if the activation of Rab5A by EGF stimulation is Rin1-dependent, HeLa cells transfected with Rab5A and scramble or Rin1-specific siRNA were subjected to R5BD-pull down assay as described above. We found that in the absence of Rin1, the activation of Rab5A in response to EGF stimulation is inhibited (Fig. 8B). These results suggest that Rab5 isoforms may be differentially regulated by various exchange factors, whose abundance, subcellular localization and responses to extra-cellular stimulation can potentially direct their favored isoforms to regulate certain pathways, but not others.

DISCUSSION

The focus of this study is to investigate the functional significance of each Rab5 isoform. Work from our lab and others has demonstrated the many facets of Rab5 in regulating endocytosis, signal transduction and cytoskeletal dynamics (36-39). Here, we provide new evidence suggesting that Rab5 isoforms have distinct capacity to coordinate the trafficking of EGFR, rather than acting only as redundant mechanisms

Rab5A is the predominant isoform in EGFR degradation pathway

After effective down-regulation of Rab5 isoforms with siRNAs, we observed that the trafficking of EGFR was delayed by Rab5A and to a lesser extent by 5B suppression, but barely affected by Rab5C silencing in both HeLa and DU145 cells. This result suggests that both endogenous Rab5A and 5B take part in EGFR trafficking, though their efficacy in mediating this process and sites of action may vary. We then looked into the expression level of endogenous Rab5 isoforms in HeLa and DU145 cells and found that Rab5A is no more enriched than Rab5B or Rab5C, indicating that the delayed degradation could not simply be attributed to overall reduction of Rab5 levels in cells. The retardation of EGFR trafficking was further analyzed by light microscopy to clarify the subcellular compartments retaining EGFR in the absence of each Rab5 isoform. We showed that EGFR entered early endosomal compartments positive for EEA1 within 10 minutes of internalization regardless of Rab5 isoform depletion. At later time points, most of the receptors had progressed out of EEA1- or Hrs-positive vesicles in control and Rab5C-depleted cells, yet remained partially localized to early endosomal compartments in Rab5A- and Rab5B-depleted cells. Though the loss of Rab5A or 5B impaired the trafficking of EGFR, it did not appear to alter the normal covalent modifications of EGFR essential for its proper sorting. Taken together, following the down-regulation of the endogenous Rab5 isoforms, we observed a differential contribution of Rab5 isoforms in EGFR trafficking. In agreement with silencing results, over-expression of Rab5A appeared to accelerate EGFR trafficking more effectively than other isoforms. In fact, previous work from our lab has also demonstrated a close connection between Rab5A and EGFR-related pathways. Barbieri *et al.* have shown that ^{125}I-EGF internalization and EGF-stimulated HRP uptake in fibroblasts are more up-regulated by over-expression of Rab5A compared with Rab5B or 5C (20). It is to be noted that the phenotypes of Rab5B suppression and overexpression did not coincide. In the absence of Rab5B, EGFR degradation is moderately impaired, but overexpression of Rab5B hardly strikes any difference. One possible explanation is that the type of cells we used for overexpression does not have sufficient and appropriate combinations of Rab5B regulators and/or effectors to transduce its activity into phenotypes; whereas, RNAi ablation of Rab5B directly perturbs the homeostasis maintained by endogenous Rab5B, which is more easily detected. We propose that endogenous Rab5B may work in conjunction or in sequence with Rab5A to facilitate the trafficking of EGFR. Interestingly, when we employed an *in vitro* system to evaluate the strength of Rab5 isoforms in promoting vesicle fusion, our preliminary data indicated that purified recombinant Rab5A was more potent in activating endosome-endosome fusion, and loss of endogenous Rab5A in cell membrane fractions lead to decreased fusion activities (data not shown). This result not only further supports the concept of differential potency from Rab5 isoforms, but also suggests that Rab5B participates in EGFR trafficking in a different manner. Recent studies point to a role of Rab5-GTP in coordinating membrane tethering and

fusion with cytoskeletal-based organelle motility (9,40) . Spatial trafficking of the early endosomes from the cell periphery to the cell center is critical for their ability to generate endosomal carrier vesicles that bud from early and fuse with late endosomes or mature into late endosomes (40,41). It is possible that Rab5B, instead of promoting endosome-endosome fusion, governs this aspect of the EGFR trafficking. In fact, we did, on multiple occasions, notice internalized EGF-EGFR vesicles to be scattered to sites more adjacent to the plasma membrane in Rab5B knockdown cells than in control or Rab5A knockdown cells. These data suggests that after EGFR is internalized, the inward movement of vesicles is likely impeded when Rab5B is repressed. More studies will be needed to clarify the involvement and activity of different Rab5 isoforms in modulating the motility of endosomes.

Rab5A preferentially interacts with Rin1, a multi-domain guanine nucleotide exchange factor that binds EGFR and regulates the EGFR degradation

The question remains unclear as to what causes the difference among Rab5 isoforms in the EGFR degradation pathway. Overexpressed Rab5 isoforms in cells have similar subcellular localization, suggesting that all three isoforms are properly targeted to membrane structures and Rab5A is more active in EGFR trafficking pathway not because of better membrane association. However, it is yet to be determined whether endogenous Rab5 isoforms are localized differently. Another possibility would be differential regulation of the isoform activities by GEFs and GAPs. At least nine different Rab5 GEFs have been identified and seven of them are proven to have GEF activity *in vitro* (7,8,32,35,42-45). In addition to vesicle fusion, Rab5 can also control the motility of organelles along microtubules (9,40), actin remodeling (37-39), and participation in proliferative cell signaling pathways (23,36). It is reasonable to speculate that the wide range of Rab5 exchange factors contribute to the complexity of these proposed functions. Indeed, we tested the binding specificity of some of these GEFs towards Rab5 isoforms, and found that Rin1 preferentially binds to Rab5A, whereas GAPex-5 showed no significant specificities towards isoforms. As we tested the activation of Rab5 isoforms in response to EGFR stimulation, Rab5A displayed strong and acute activation, which is in good correlation with its potency in EGFR trafficking. More importantly, the activation of Rab5A upon EGF stimulation is inhibited by silencing of Rin1. That is, the preferential interaction between Rab5A and Rin1 appears to control the ability and sensitivity of Rab5A in regulating EGFR degradation pathway in HeLa cells. However, it remains unclear whether the preferential interaction between Rin1 and Rab5A selectively regulates the trafficking of only a certain receptors. Barbieri *et al.* showed that Rin1 associates with several signaling receptors, but not cargo receptors, such as Tfn receptor and Mannose receptor (34). Moreover, overexpression of Rin1 does not alter the uptake of Tfn, nor was the recycling of Tfn in HeLa cells affected by Rin1 depletion (Fig.S7). These results suggest that Rin1 may provide selectivity for Rab5A in regulation of the trafficking of specific receptors.

As more functions have been shown to involve Rab5, it is possible that different Rab5 isoforms play more significant roles in distinct functions. In fact, preliminary studies examining Rab5-mediated cell migration implicates Rab5C to be more engaged to cell migratory activities than other Rab5 isoforms (Chen and Stahl, unpublished data). In agreement with this finding, a recent report indicated that Rab5C is the only isoform expressed during gastrulation of zebrafish and mediates Wnt11-controlled mesendodermal cell cohesion and migration (46). Ulrich *et al.* proposed that Wnt11 promotes Rab5C-mediated endocytosis and recycling of E-cadherin to regulate the dynamics of E-cadherin turnover at the plasma membrane. Alternatively, Wnt11 might regulate Rab5-dependent actin remodeling, which in turn could affect the adhesive and cohesive properties of mesendodermal cells.

It is important to point out that lower eukaryotic model organisms such as *Drosophila* and *C. elegans* have only one Rab5 paralogue, and loss of its function has been shown to result in embryonic lethality (47,48); whereas, three isoforms are identified for higher organisms (i.e. human and zebrafish). The multiplication of Rab5 paralogues from its ancestral origin implicates continued evolution and specialization of the early endosomal systems. The most defining biological attributes of higher organisms are the development

of nervous and immune systems, both of which require highly organized endocytic networks in order to manage the intricacy of their respective functions. In the nervous system, not only Rab5 is shown to associate with vesicles involved in cycling of neurotransmitters during synaptic transmission (47), but its activity appears to initiate neuronal cell differentiation and dendrite branching (33,49,50), and participates in the delivering of neurotrophic "signaling endosomes" via dynein and kinesin (50,51). Several neurodegenerative diseases are linked to faulty axonal transport (52). The juvenile amyotrophic lateral sclerosis-associated gene ALS2, encoding the protein Alsin, whose Vps9 domain regulates the activation of Rab5 (43). Depletion mutation of ALS2 within its Vps9 domain has been identified to cause hereditary spastic paraplegia (53), further stressing the role of Rab5 in neuronal pathogenesis. Recent evidence also revealed multiple endocytic mechanisms governing antigen presentation (54). Depending upon the nature of the antigens as well as the endocytosis receptors, antigens can be routed to phagosomes or endosomes for peptide processing and loading. The molecular basis that assigns each Rab5 isoform to a specific function is still unclear. In this study, we provided new evidence suggesting that Rab5 isoforms have preferences towards their exchange factors and certain pathways. Given the need for flexible yet precise regulation of these neuronal and immune responses, it is reasonable to anticipate that Rab5 isoforms differentially integrate their binding specificities and/or tissue distribution into the regulation of multiple pathways. It will be of great importance to dissect the roles of Rab5 isoforms in these pathways by means of individual Rab5 isoform ablation in the future.

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FOOT NOTES

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The abbreviations used are: Rin1, Ras interaction/interference1; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; STAM, signal transducing adapter molecule; EEA1 early endosomal antigen 1; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; ESCRT, endosomal sorting complex required for transport; siRNA, short interfering RNA; GFP, green fluorescent protein; SH, Src homology; GEF, Guanine nucleotide exchange factor; GAP, GTPase activating protein.

FIGURE LEGENDS

Figure 1. Depletion of individual Rab5 isoforms differentially delayed the degradation of EGFR in HeLa and DU145 cells

A) HeLa cells were transfected with 20 nM siRNA targeting GFP (as negative control) or specific sequences of the Rab5 isoforms. 48 hours post-transfection, cells were harvested and then subjected to SDS-PAGE and immunoblot analysis. Proteins were probed with specific antibodies as indicated. B) HeLa cells transfected with siRNA targeting GFP (as negative control), Rab5A, Rab5B or Rab5C were

starved for 2-3 hours and degradation of EGFR was stimulated with 100 ng/ml EGF for the indicated times. Cell lysates were subjected to SDS-PAGE analysis. Proteins were probed with specific antibodies as indicated. C) Band intensity from 15, 30 and 60 minutes was quantified with AlphaEaseFc 4.0 software. The graph was acquired from three independent experiments. The data represent the mean value ± S.E. * $P<0.007$ compared to control. D) DU145 cells were transfected with siRNA targeting GFP (as negative control), Rab5A, Rab5B or Rab5C. 24 hours post-transfection, cells were starved overnight and degradation of EGFR was stimulated with 100 ng/ml EGF in the presence of cycloheximide for 5 hours. Cell lysates were processed as described above. Proteins were probed with specific antibodies as indicated. Band intensity was quantified with AlphaEaseFc 4.0 software. The graph was acquired from three independent experiments. The data represent the mean value ± S.E. * $P<0.006$ compared to control

Figure 2. Determination of the abundance of endogenous Rab5 isoforms in HeLa and DU145 cells

5 or10 ug of total cell lysates from siRNA-treated DU145 or HeLa cells were subjected to SDS-PAGE analysis alongside purified His-Rab5 isoforms ranging from 1.25 ng to 10 ng as standards. Proteins were probed with isoform specific antibodies and developed with ECL. Band intensity was quantified with AlphaEaseFc 4.0 software (supplementary figure 2). A) The experiment was repeated three times. The graph shows the relative abundance of the three isoforms in HeLa cells. The data represents the mean value of three independent experiments ± SE. B) The abundance of Rab5 isoforms in DU145 was determined as described above. The graph shows the relative abundance of the three isoforms in DU145 cells. The data represent the mean value of three independent experiments ± SE.

Figure 3. Over-expression of Rab5A accelerates the trafficking of EGFR

A) HeLa cells were transfected with CFP, CFP-Rab5A, 5B, or 5C. 24 hours post-transfection, cells were starved and then subjected to EGFR degradation assay. Cell lysates were analyzed by SDS-PAGE. Proteins were probed with specific antibodies as indicated. B) Band intensity was quantified with AlphaEaseFc 4.0 software. The graph was acquired from three independent experiments. The data represent the mean value ± SE. C) HeLa cells were transfected with RFP-Rab5A, Rab5B, or Rab5C. 24 hours post-transfection, cells were fixed and immunostained with EGFR antibody to determine the level of EGFR in cells. Scale bar represents 5 μm

Figure 4. Internalization of EGFR and Transferrin receptors are not inhibited by individual Rab5 isoform depletion

A) The rate of EGFR internalization was examined by labeling cell surface EGFR with biotin after stimulation briefly with EGF. Magnetic streptavdin beads then were used to pull-down biotin labeled surface EGFR. After washes, bound EGFR were released from the beads with SDS sample buffer, and then analyzed with SDS-PAGE. B) The intensity of the bands were quantified with AlphaEaseFc 4.0 software and plotted as percentage of EGFR remaining on cell surface over time. The data represent the mean value of three independent experiments ± SE. C) Control or specific-siRNA transfected cells were starved for one hour to deplete endogenous Tfn before the uptake assay. Next, cells were pre-bound with Alexa-488-Tfn for 1 hour at 4°C. Internalization was then initiated by replacing the binding medium with pre-warmed internalization medium and incubating at 37°C for 5 minutes. At the end of uptake, cells were washed and fixed for confocal microscopy analysis. Scale bar represents 10 μm

Figure 5. Phosphorylation and ubiquitylation of EGFR is not altered by silencing of Rab5 isoforms

A) Control or Rab5 isoform-silenced HeLa cells were starved and stimulated with EGF at 37°C for the indicated times. Cell lysates were then subjected to EGFR immunoprecipitation and immunoblotting with pan-ubiquitin antibody to detect the ubiqitylated EGFR. Blots were re-probed with EGFR antibody to show the total EGFR levels in the immunoprecipitates. B) Control or Rab5 isoform-silenced cells were

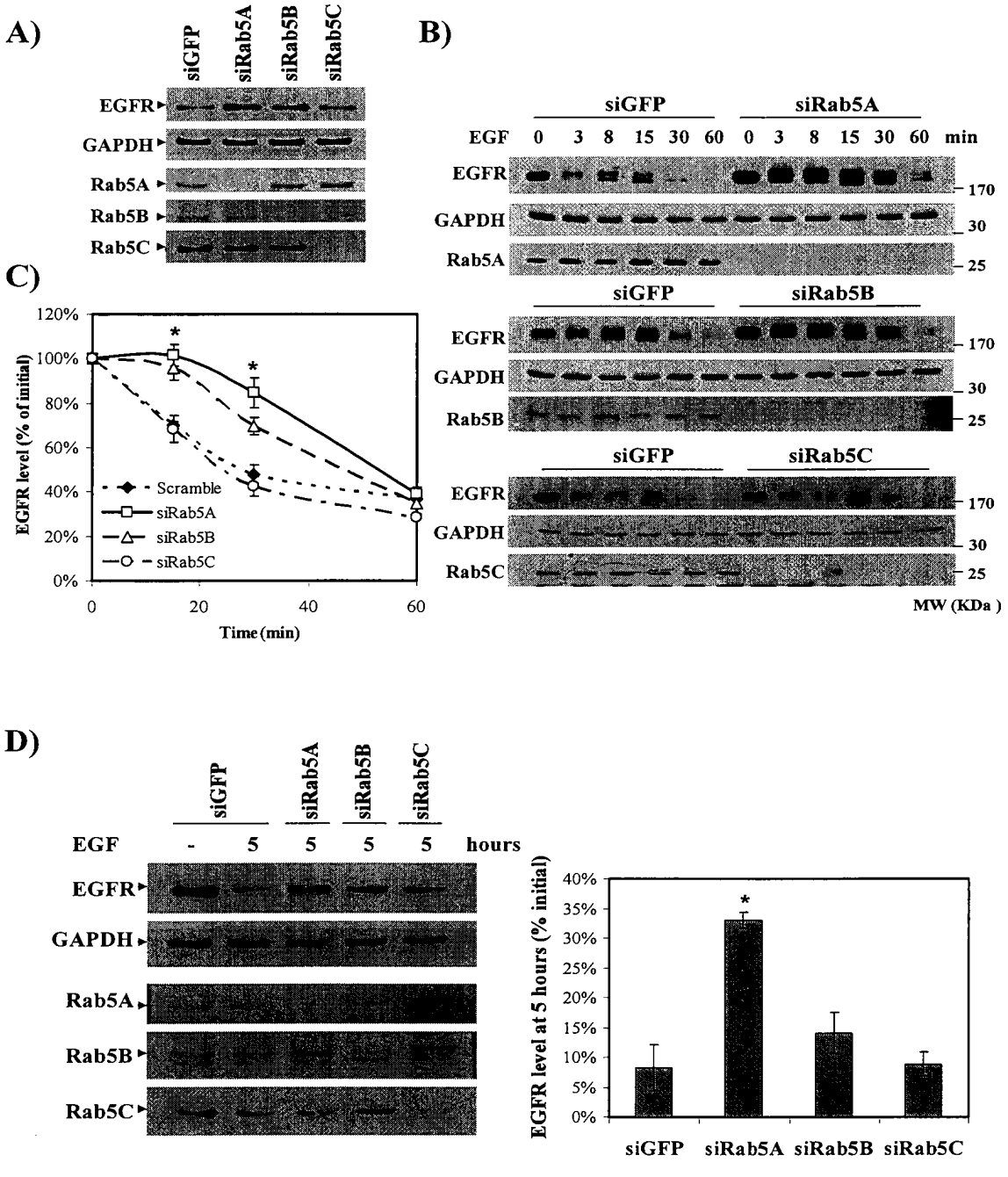
starved and then stimulated with EGF at 4 °C for one hour. Cell lysates were then subjected to EGFR immunoprecipitation and immunoblotting with antibodies as indicated. Co-immunoprecipitated adaptor proteins were detected with specific antibodies as indicated.

Figure 6. Trafficking of EGFR into EEA1-positive compartments is not altered by depletion of Rab5 isoforms, but exit from early endosomal compartments is delayed
HeLa cells were transfected with control or Rab5 isoform-specific siRNA. 48 hours post-transfection, cells were starved, incubated with Alexa488-EGF for 1 hour at 4 °C, and then allowed to endocytose for A) 10 minutes, and B) 25 minutes at 37 °C. Cells were then fixed and immunostained for EEA1 (2° Ab: Alexa594-IgG). Scale bar represents 10 μm C) Colocalization between EGF/EGFR and EEA1 was quantified with ImageJ and the mean value of the Pearson's coefficient ± SE from 7 images per condition is illustrated in the graph.

Figure 7. Rab5A is activated in response to EGF stimulation
HeLa cells were transfected with GFP-Rab5A, 5B or 5C. 24 hours post-transfection, cells were starved for 3 hours and then stimulated with EGF for 5 minutes. Cell lysates were collected and subjected to R5BD-GST pull down assay as described in Experimental Procedure. Bound GFP-Rab5 isoforms were then analyzed with SDS-PAGE and immunoblotting. B) Band intensity from blots was quantified, and normalized to total GFP-Rab5 isoform signals from whole cell lysates (WCL). The adjacent graph represents mean ± SE of the relative activation of Rab5 isoforms from three independent experiments.
* $P < 0.04$

Figure 8. Rin1 preferentially binds Rab5A to facilitate the degradation of EGFR
A) HEK293 cells were co-transfected with V5-tagged Rin1 along with constitutively active (Q79L) or negative (S34N) mutants of GFP-Rab5A, Rab5B or Rab5C. Cell lysates were pre-cleared with centrifugation and then immuno-precipitated with anti-GFP antibody. Immunoprecipitates were then separated by SDS-PAGE and blotted with antibodies as indicated above. Total lysates were also analyzed to show the expression level of recombinant proteins in each sample. B) HeLa cells were co-transfected with GFP-Rab5A and scramble- or Rin1-targeting siRNA. 24 hours post-transfection, cells were starved for 3 hours and then stimulated with EGF for 5 minutes. Cell lysates were collected and subjected to R5BD-GST pull down assay as described in Experimental Procedure. Bound GFP-Rab5 isoforms were then analyzed with SDS-PAGE and immunoblotting. C) Band intensity from blots was quantified, and normalized to total GFP-Rab5A signals from WCL. The adjacent graph represents mean ± SE of the relative activation of Rab5A from three independent experiments.

Figure 1


A)
siGFP
siRab5A
siRab5B
siRab5C
EGFR
GAPDH
Rab5A
Rab5B
Rab5C
B)
siGFP
siRab5A
EGF 0 3 8 15 30 60 0 3 8 15 30 60 min
EGFR
GAPDH
Rab5A
170
30
25
siGFP
siRab5B
EGFR
GAPDH
Rab5B
170
30
25
siGFP
siRab5C
EGFR
GAPDH
Rab5C
170
30
25
MW (KDa)
C)
EGFR level (% of initial)
120%
100%
80%
60%
40%
20%
0%
Scramble
siRab5A
siRab5B
siRab5C
0
20
40
60
Time (min)
D)
siGFP
siRab5A
siRab5B
siRab5C
EGF - 5 5 5 5 hours
EGFR
GAPDH
Rab5A
Rab5B
Rab5C
EGFR level at 5 hours (% initial)
40%
35%
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siRab5B
siRab5C

Figure 2

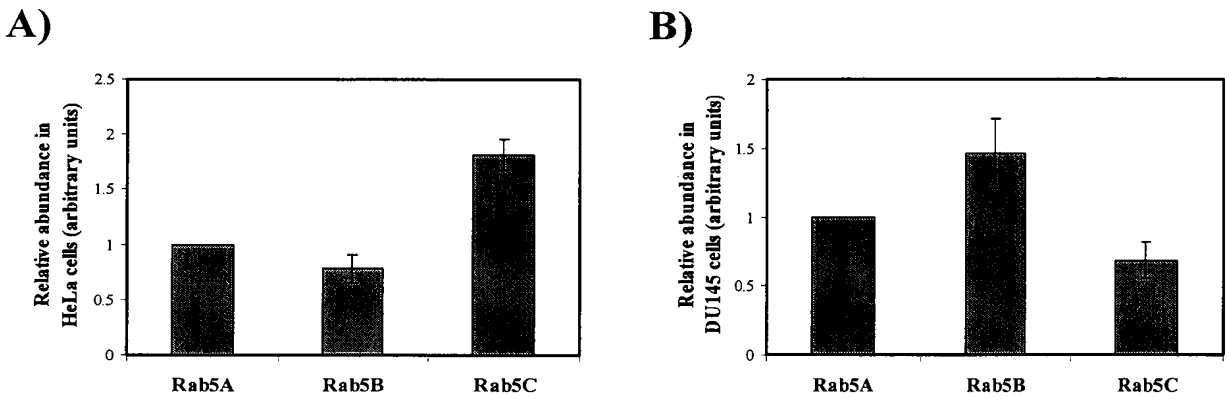

A)
Relative abundance in HeLa cells (arbitrary units)
2.5
2
1.5
1
0.5
0
Rab5A
Rab5B
Rab5C
B)
Relative abundance in DU145 cells (arbitrary units)
2
1.5
1
0.5
0
Rab5A
Rab5B
Rab5C

Figure 3

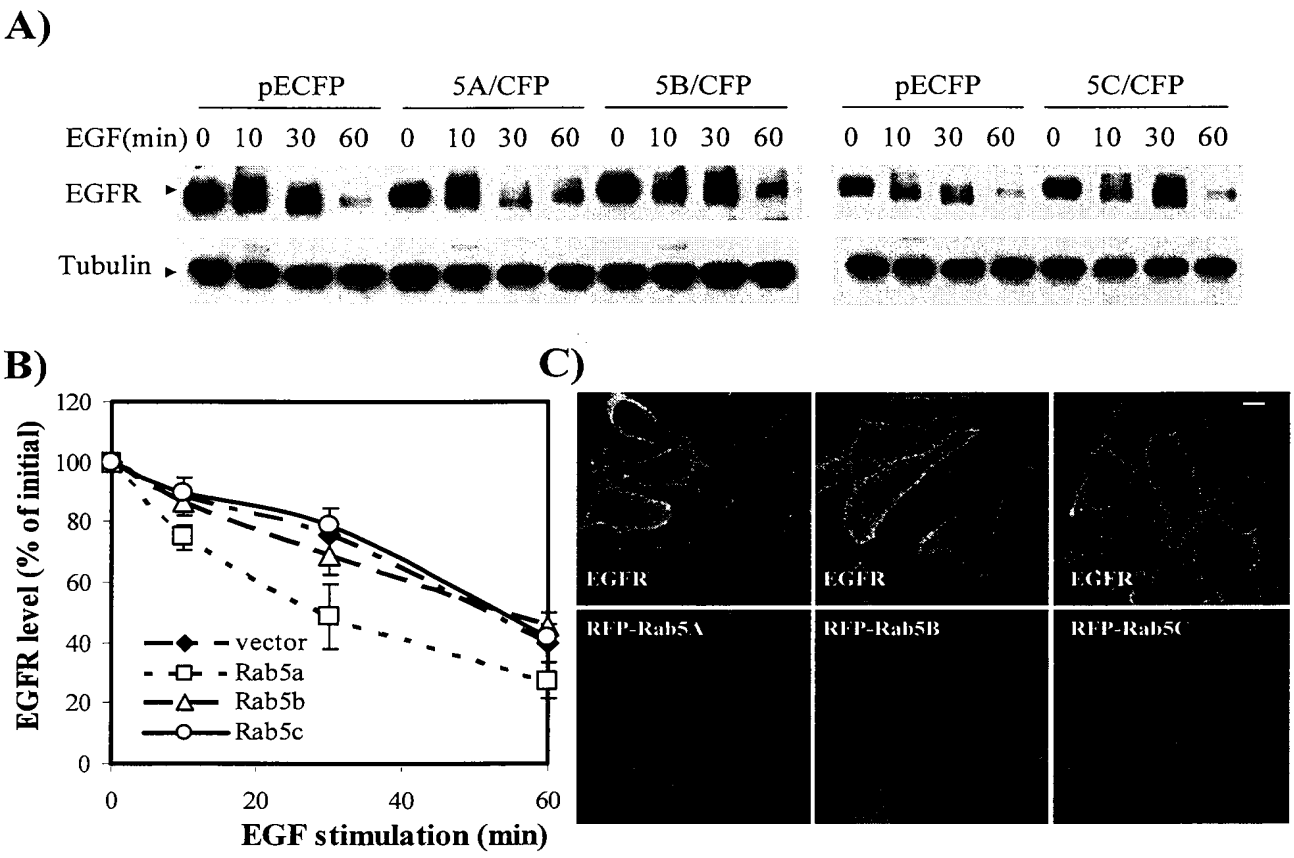

A)
pECFP
5A/CFP
5B/CFP
pECFP
5C/CFP
EGF(min) 0 10 30 60 0 10 30 60 0 10 30 60 0 10 30 60 0 10 30 60
EGFR
Tubulin
B)
EGFR level (% of initial)
120
100
80
60
40
20
0
0
20
40
60
EGF stimulation (min)
vector
Rab5a
Rab5b
Rab5c
C)
EGFR
EGFR
EGFR
RFP-Rab5A
RFP-Rab5B
RFP-Rab5C

Figure 4

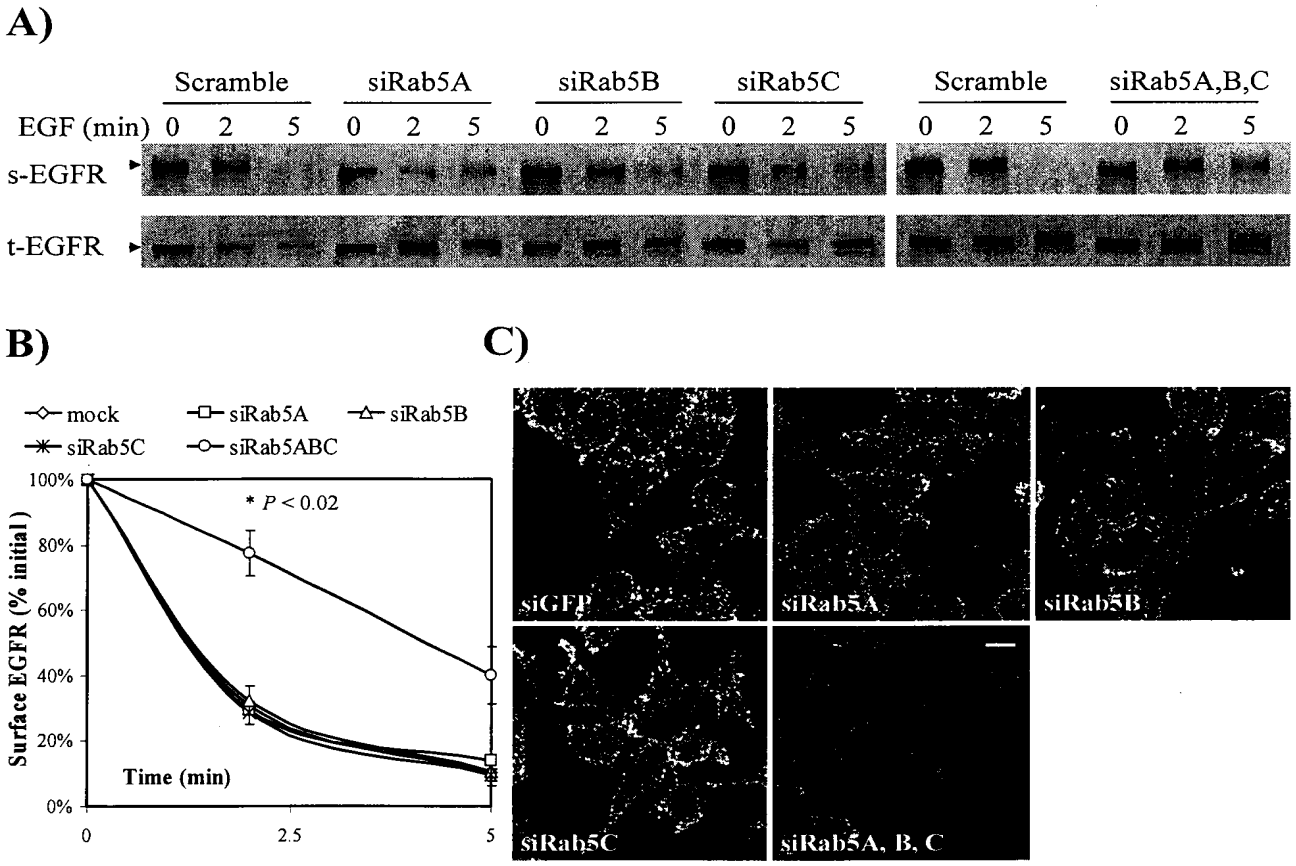

A)
Scramble
siRab5A
siRab5B
siRab5C
Scramble
siRab5A,B,C
EGF (min) 0 2 5 0 2 5 0 2 5 0 2 5 0 2 5 0 2 5
s-EGFR
t-EGFR
B)
mock
siRab5A
siRab5B
siRab5C
siRab5ABC
* P < 0.02
Surface EGFR (% initial)
100%
80%
60%
40%
20%
0%
Time (min)
0
2.5
5
C)
siGFP
siRab5A
siRab5B
siRab5C
siRab5A, B, C

Figure 5

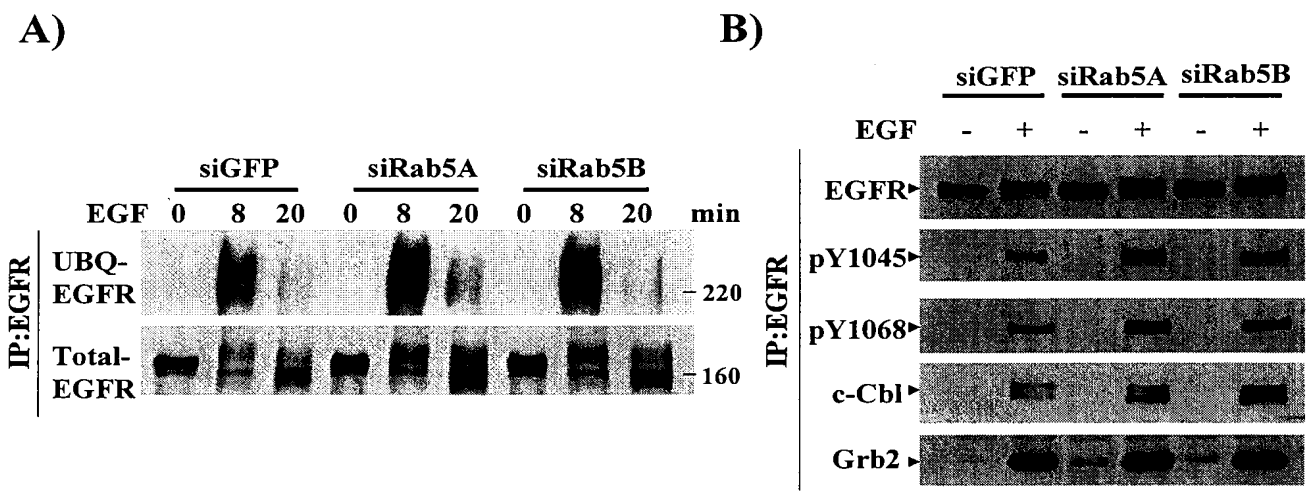

A)
siGFP
siRab5A
siRab5B
EGF 0 8 20 0 8 20 0 8 20 min
IP:EGFR
UBQ-EGFR
Total-EGFR
220
160
B)
siGFP siRab5A siRab5B
EGF - + - + - +
IP:EGFR
EGFR
pY1045
pY1068
c-Cbl
Grb2

Figure 6

A) Alexa488-EGF Uptake: 10 min

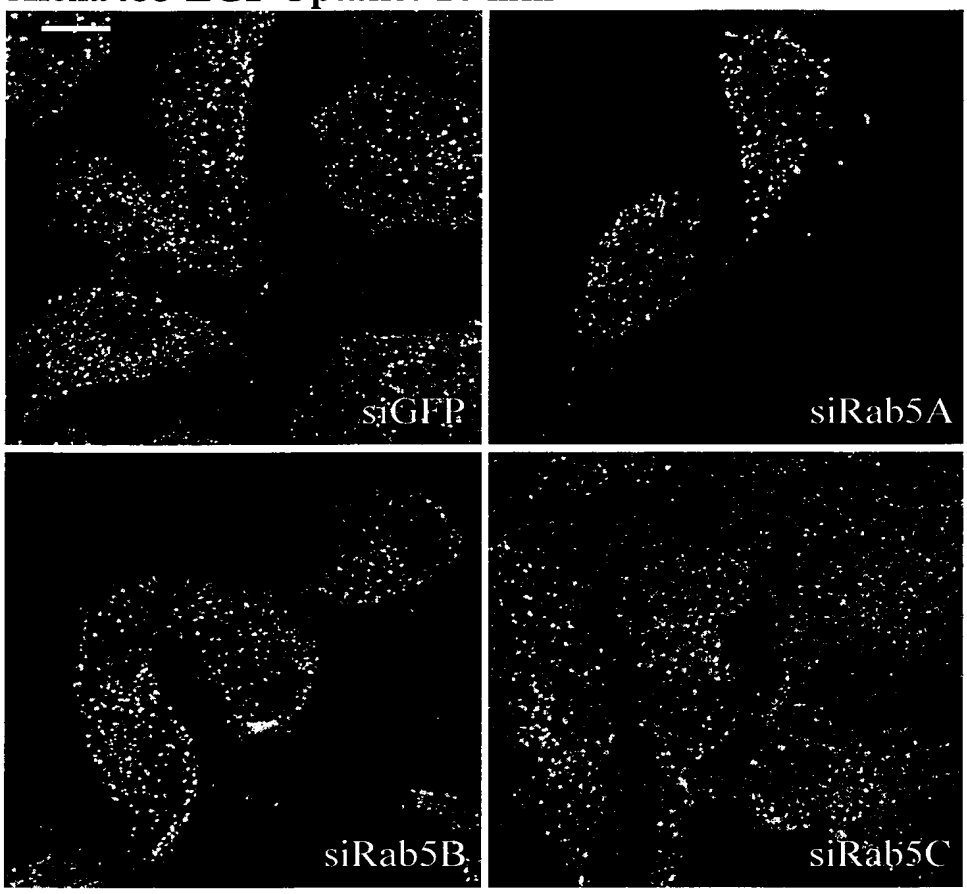


Figure 6

B) Alexa488-EGF Uptake: 25 min

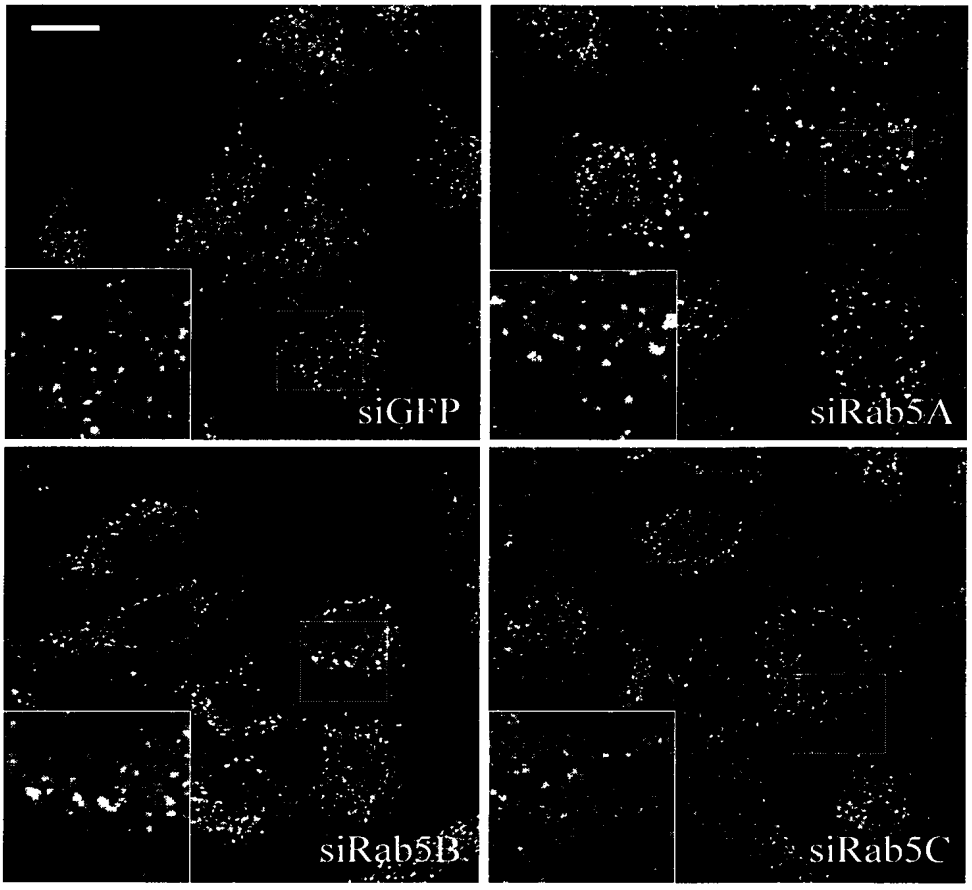


Figure 6

C)

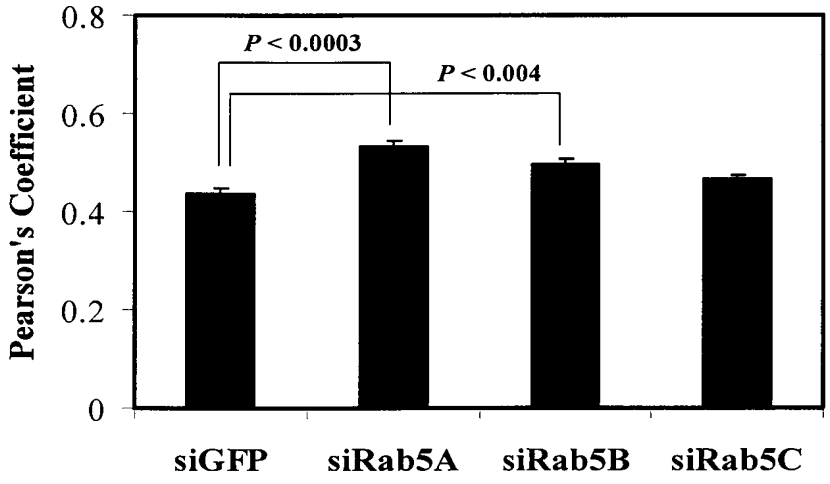

0.8
0.6
0.4
0.2
0
Pearson's Coefficient
P < 0.0003
P < 0.004
siGFP
siRab5A
siRab5B
siRab5C

Figure 7

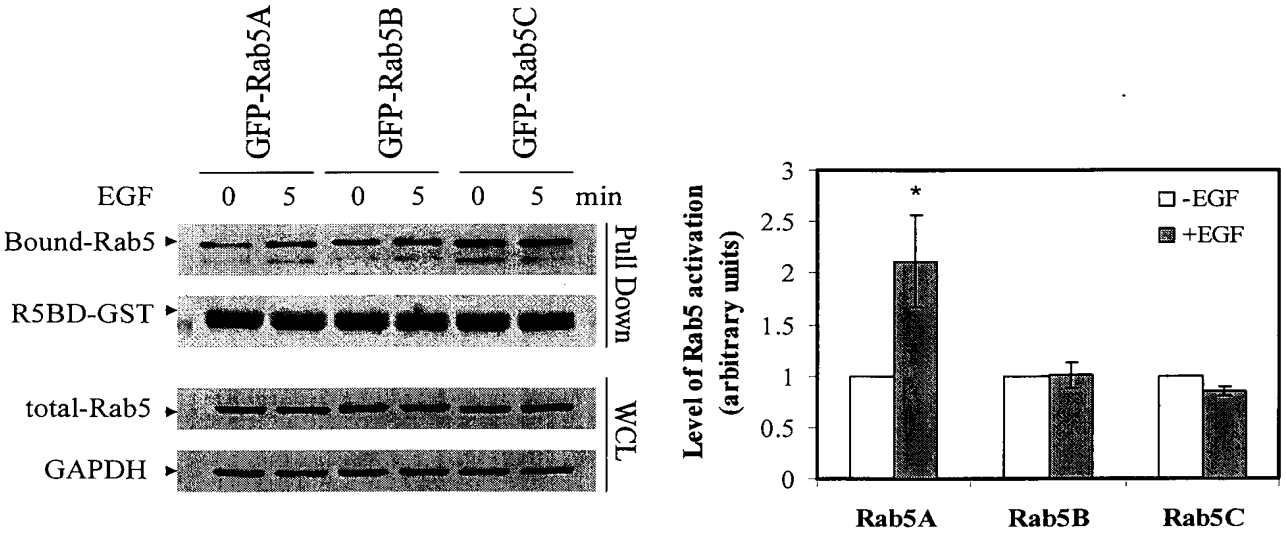
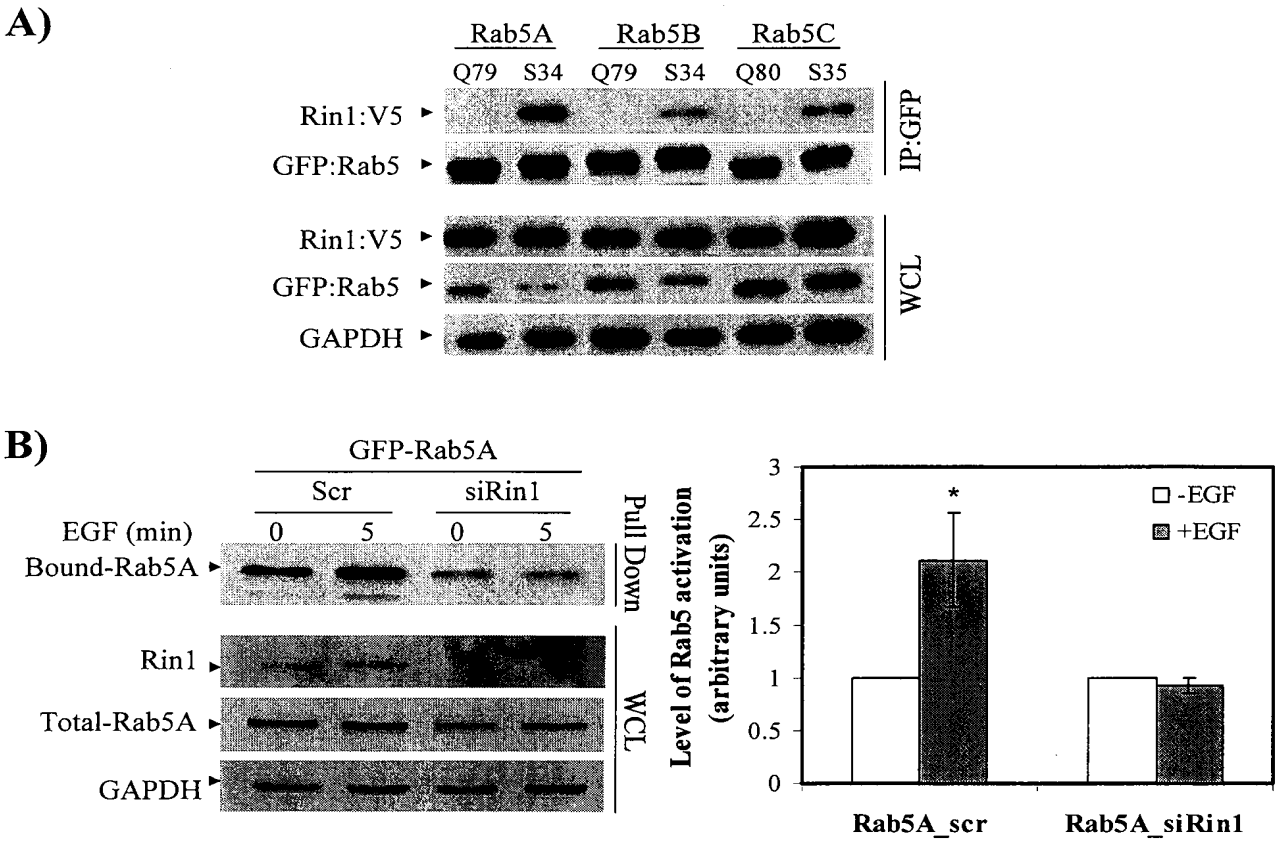

GFP-Rab5A
GFP-Rab5B
GFP-Rab5C
EGF
0 5 0 5 0 5 min
Bound-Rab5
R5BD-GST
Pull Down
total-Rab5
GAPDH
WCL
Level of Rab5 activation (arbitrary units)
3
2.5
2
1.5
1
0.5
0
*
-EGF
+EGF
Rab5A
Rab5B
Rab5C

Figure 8


A)
Rab5A
Rab5B
Rab5C
Q79
S34
Q79
S34
Q80
S35
Rin1:V5
GFP:Rab5
IP:GFP
Rin1:V5
GFP:Rab5
GAPDH
WCL
B)
GFP-Rab5A
Scr
siRin1
EGF (min)
0
5
0
5
Bound-Rab5A
Pull Down
Rin1
Total-Rab5A
GAPDH
WCL
Level of Rab5 activation (arbitrary units)
3
2.5
2
1.5
1
0.5
0
*
-EGF
+EGF
Rab5A_scr
Rab5A_siRin1